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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	John Haurum et al.	Art Unit:	1644
Serial No.:	09/866,573	Examiner:	Margaret E. Jamroz
Filed:	May 25, 2001	Customer No.:	21559
Title:	A POLYCLONAL ANTIBODY COMPOSITION FOR TREATING ALLERGY		

Assistant Commissioner For Patents
Washington, DC 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF JOHN S. HAURUM, M.D., Ph.D.

1. I, John S. Haurum, am an inventor on the above-captioned patent application. My qualifications include extensive research experience in molecular immunology and biochemistry, with 25 peer-reviewed and published articles in international scientific journals.
2. I have read the Office Action mailed March 7, 2002, filed in the parent case, U.S.S.N. 09/866,573, and submit the following declaration in support of the present application.
3. Acquired hypersensitivity through the exposure of a particular allergen is, in some cases, mediated by entry of the allergen into the body. In most cases, entry of an allergen into the body requires an allergen to traverse a mucosal surface. The surface mucosa is at the interface between the external environment and internal body. Innate defense mechanisms reside at the mucosal surface, for example, entrapment of particles in mucus

immobilizes and facilitates clearance of the particle by peristalsis, ciliary movement, or the flow of fluids across the epithelial layer to flush away the particle. As such, transport of immunoglobulins from the luminal surface into the body is not necessary for the immunoglobulins to inhibit or reduce an allergic reaction to an allergen present on the luminal side of the mucosa. For example, polyclonal antibodies specific to an allergen would be able to bind to and aggregate the allergen, thus preventing passive or active transport of the allergen across the mucosal surface into the body. Because of the polyclonal nature of the antibodies, more epitopes would be recognized resulting in larger (size and number) immune-complex formation. Furthermore, as an aggregate, entrapment and clearance of the allergen-polyclonal antibody complexes are more readily instigated.

4. While it has been well established that polymeric immunoglobulins (IgA and IgM) can cross the mucosal surfaces by way of the polymeric immunoglobulin receptor (pIgR), monomeric immunoglobins have also been shown to cross epithelial surfaces. Of particular note is the enclosed document, Dickenson *et al.* (J. Clin. Invest. 104:903-911, 1999; herein referred as Dickenson). Dickenson teaches that monomeric IgG can be transported across epithelial surfaces. This transport is mediated by the Fc receptor, FcRn, and transport (transcytosis) is bi-directional. Furthermore, contrary to previous reports, FcRn is not restricted in expression to neonates, but is also clearly expressed in adult epithelial tissue.

5. Thus, the use of polyclonal antibodies for treating allergies can be effected at the luminal surfaces of the respiratory and gastrointestinal tract, as well as the conjunctival and urogenital mucosa. At these mucosal surfaces, antibodies can bind to their corresponding allergens, thereby aggregating or chelating the allergens, preventing entry into the systemic circulation, and the allergens eventually being cleared away. This is accomplished without traversing the mucosal membrane, and without eliciting a local

allergic reaction on the luminal side, due to the blocking of access of all local allergen-specific IgE. Conversely, polyclonal antibodies in the systemic circulation would also bind onto their corresponding allergens and effect clearance by the classical immune pathways described in the application. Treatment of allergies would not necessitate the transport of the antibodies into the systemic circulation. In fact, it is desirable to prevent allergen transport across the mucosal surface.

6. Clinical strategies for antibodies employed in cancer treatment, requires activation of the host immune system. Polyclonal antibodies that are directed to tumor antigens on a tumor cell would facilitate effector functions of antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Tumor cells bound to polyclonal antibodies would induce ADCC by natural killer (NK), cytotoxic T cells, neutrophils, macrophage, or any effector cell expressing an Fc γ receptor on its surface. It should be noted that ADCC would be facilitated only in the presence of antibodies of the IgG isotype, due to the requirement of the Fc γ receptor.

7. Complement dependent cytotoxicity (CDC) is also effected by antibodies bound to tumor cells. In the classical complement pathway, antibodies bound to the tumor cell surface are recognized and bound by the serum C1 protein. Serum C1 is a protein complex composed of three proteins: C1q, which binds to the Fc region of the antibody, C1s, a protease that cleaves the next complement component (C4), and C1r, an adaptor molecule bridging C1q to C1s. The C1-antibody interaction results in the sequential activation of complement components, C4, C2, and C3, leading to the formation of cleavage enzymes, C3 and C5 convertase. The cascade of events is completed with the activation of C5, C6, C7, C8, and C9, to form the membrane attack complex (MAC), which go on to lyse the tumor cell. The activation of C3 and C5 components results in formation of C3a, C3b, C5a, and C5b. C3a and C5a have anaphylactic activity. C5a is further, a powerful chemoattractant of neutrophils and monocytes.

8. Thus, activation of ADCC and CDC further results in the activation of an inflammatory response. The inflammatory pathway is mediated by both the adaptive and non-specific immune responses to recruit more effector cells to the proximity of the tumor cell. Activated components of the complement system are able to mediate inflammation through the recruitment of effector cells by acting as chemoattractants (C5a), by acting as anaphylaxins (mast cells and basophils express C3a and C5a receptors, releasing histamines when activated by the corresponding ligands), or by promoting increased capillary permeability (C4a). Furthermore, various cytokines are released to further recruit effector cells, for example, C5a binding to macrophages results in interleukin-1 production, which promotes the growth and activity of a variety of immune cells.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date:

6th June 2002
John S. Haurum, M.D., Ph.D.

21559

PATENT TRADEMARK OFFICE

Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line

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The MHC class I-related Fc receptor, FcRn, mediates the intestinal absorption of maternal IgG in neonatal rodents and the transplacental transport of maternal IgG in humans by receptor-mediated transcytosis. In mice and rats, expression of FcRn in intestinal epithelial cells is limited to the suckling period. We have recently observed, however, clear expression of FcRn in the adult human intestine, suggesting a function for FcRn in intestinal IgG transport beyond neonatal life in humans. We tested this hypothesis using the polarized human intestinal T84 cell line as a model epithelium. Immunocytochemical data show that FcRn is present in T84 cells in a punctate apical pattern similar to that found in human small intestinal enterocytes. Solute flux studies show that FcRn transports IgG across T84 monolayers by receptor-mediated transcytosis. Transport is bidirectional, specific for FcRn, and dependent upon endosomal acidification. These data define a novel bidirectional mechanism of IgG transport across epithelial barriers that predicts an important effect of FcRn on IgG function in immune surveillance and host defense at mucosal surfaces.

J. Clin. Invest. 104:903–911 (1999).

Introduction

Steady-state levels of IgG in the blood of adult mice, and likely all mammals, depend on IgG catabolism mediated in part by the MHC class I-related Fc receptor, FcRn (1). FcRn also mediates vectorial transport of IgG across certain epithelial barriers. In suckling mice and rats, intestinal absorption of maternal IgG from breast milk into the systemic circulation depends on FcRn (2). In humans, maternofetal transfer of IgG across the placenta also likely depends on FcRn (3). Thus, FcRn plays critical and well-documented roles in the regulation of IgG metabolism in adults and in the acquisition of humoral immunity in early life. These effects on the physiology of IgG in vivo result from the action of FcRn as an intracellular trafficking receptor (4).

FcRn has been cloned from the rat, mouse, and human. The molecule is expressed as a heterodimer composed of a glycosylated heavy (α) chain (51 kDa in rodents and 40–45 kDa in humans) associated noncovalently with β_2 -microglobulin (β_2 M) (5). Binding of IgG to FcRn requires contact between solvent-exposed

peptide sequences in the CH2 and CH3 domains of IgG and the α 1 and α 2 domains of FcRn, together with a single contact site in β_2 M (6–11). A hallmark of FcRn interaction with IgG is its pH dependence, showing high-affinity binding at acidic pH ($\text{pH} \leq 6.5$) and weak or no binding at neutral pH ($\text{pH} \geq 7.0$) (12, 13). FcRn is the only Fc γ receptor that exhibits MHC class I structure, and the only Fc γ receptor to exhibit pH dependency in ligand binding.

The function of FcRn in the intestine of suckling mice and rats has been well documented (14). In neonatal mice and rats, FcRn is expressed at high levels by intestinal epithelial cells and mediates absorption of IgG by receptor-mediated transcytosis. FcRn expression in the neonatal rodent is developmentally downregulated, resulting in nearly complete loss of intestinal FcRn at the time of weaning (15–17). Consequently, a role for FcRn beyond neonatal life has been slow to emerge. However, we have recently observed expression of FcRn in adult rat hepatocytes (18) and in adult human intestinal epithelial cells (19). The FcRn detected in isolated human small

intestinal epithelial cells had identical predicted amino acid sequence to that expressed by human placenta (20). These data raised the possibility that FcRn may function to transport IgG across the adult human intestinal epithelium.

In this study, we show that the polarized human intestinal epithelial cell line T84 transports IgG by receptor-mediated transcytosis. Transport is bidirectional and dependent on FcRn. These data define the function of FcRn in a model intestinal epithelial cell line, and indicate that FcRn may function *in vivo* to transport IgG across the epithelial barrier of the intestine and possibly across other mucosal surfaces of the adult human. As such, these data predict a profound effect of FcRn on IgG function in immune surveillance and host defense.

Methods

Cell lines and human tissue. Human cell lines T84, HT29, Caco-2, CEM, 293, U937, CHO, and MOLT-4 were purchased from American Type Culture Collection (Rockville, Maryland, USA). Normal adult human small intestine was obtained from patients undergoing gastric bypass surgery, and epithelial cells were nonenzymatically isolated as described (21, 22). T84 cells (passages 28–61; gift from K. Barrett, University of California San Diego, San Diego, California, USA) were grown on Transwell inserts (Corning-Costar Corp., Cambridge, Massachusetts, USA) as described previously (23). Transcytosis experiments were performed in HBSS supplemented with 10 mM HEPES (pH 7.4) (HBSS+) (Sigma Chemical Co., St. Louis, Missouri, USA). T84, Caco-2, or HT29 cells were cell surface biotinylated or metabolically labeled as described previously (24, 25).

Antibodies. Anti-FcRn antibodies purified from 2 rabbit antipeptide antisera were used, one raised against amino acids 112–125 of human FcRn (3), the other against amino acids 174–188 (K. McCarthy and N.E. Simister, manuscript in preparation). Polyclonal antisera were also raised in mice against the $\alpha 2$ domain of human FcRn expressed as a GST fusion protein in *Escherichia coli*. These antisera did not cross-react with either MHC class I or CD1d, as defined by Western blotting of 721.220, 721.220-CD1d transfectant, Jurkat, THP1, and HeLa cells. HeLa cells cotransfected with FcRn α chain and β_2 M expression plasmids were used as positive controls (data not shown).

Western and Northern blots. T84, HT29, Caco-2, 293 cells transfected with a cDNA-encoding human FcRn α chain (20), untransfected 293 cells, and normal human small intestinal cells (19) were extracted in 5% SDS in water. Protein extracts were resolved on gradient polyacrylamide denaturing Tris-glycine gels (Novex, San Diego, California, USA) and transferred onto PVDF (Novex) or nitrocellulose (Bio-Rad Laboratories Inc., Hercules, California, USA). Blots were probed with affinity-purified anti-FcRn peptide antibody, and bound antibody was detected with horseradish peroxidase-conjugated goat anti-rabbit anti-

body and enhanced chemiluminescence (Renaissance Chemiluminescence Reagent; NEN Life Science Products Inc., Boston, Massachusetts, USA). For deglycosylation studies, protein extracts (10 μ g total protein) prepared in 5% SDS in water were deglycosylated with PNGase F (New England Biolabs Inc., Beverly, Massachusetts, USA), separated by SDS-PAGE under reducing and nonreducing conditions, and blotted for FcRn as already described here.

For Northern blots, total cellular RNA was extracted from T84, HT29, Caco-2, CEM, 293-FcRn, 293 cells, and normal human small intestinal cells using Ultraspec (Biotech Laboratories Inc., Houston, Texas, USA). RNA (10 μ g/lane) was run on a denaturing agarose gel and transferred to a Biotrans Nylon Membrane (ICN Radiochemicals Inc., Irvine, California, USA). The blot was hybridized with 2×10^6 cpm/mL of 32 P-labeled probe derived from the 120-bp *Xmn*-*Eco*RI fragment of a human placental FcRn cDNA clone (3). The blot was hybridized in QuikHyb (Stratagene, La Jolla, California, USA), washed, exposed on a phosphor screen (Eastman Kodak Co., Rochester, New York), and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA).

RT-PCR and nested PCR. Total RNA was isolated from T84, MOLT-4, and U937 cells with Trizol reagent (GIBCO BRL, Gaithersburg, Maryland, USA). RNA (2 μ g) was reverse-transcribed to cDNA with an oligo-dT primer (Promega Corp., Madison, Wisconsin, USA) and avian myeloblastosis virus reverse transcriptase (Promega Corp.). The cDNA samples were used as a template in a nested PCR reaction, performed to increase amplification of Fc γ RI transcripts in resting monocytes as described previously (26). First- and second-round primers specific for Fc γ RI cDNA were identical to those designed by van de Winkel et al. (27). The first-round PCR product (30 cycles) was diluted 1:2.5 and amplified with nested primers specific for Fc γ RI or with primers specific for β -actin. In addition to the major 800-bp Fc γ RI product, 2 other transcripts (~700 and 1,000 bp) were amplified, likely reflecting the multiple transcripts for Fc γ RI observed in human monocytes (26, 28).

Immunohistochemistry and confocal microscopy. Normal human small intestine and polarized T84 cell monolayers were embedded in Tissue-Tek OCT Compound (Sakura-Finetek Inc., Torrance, California, USA) for frozen sectioning on a Leica CM3050 cryomicrotome (Leica, Nussloch, Germany). Frozen sections (5 μ m) were air-dried at room temperature, fixed in 4% paraformaldehyde in PBS, washed in PBS, and blocked in 10% normal goat serum (Zymed Laboratories Inc., South San Francisco, California, USA). Sections were stained with an affinity-purified rabbit anti-human FcRn peptide antibody (amino acids 174–188) or a mouse polyclonal anti-FcRn antiserum diluted in the blocking solution and detected with appropriate fluorophore-conjugated secondary antibodies for epifluorescence microscopy. For some experiments, T84 cells grown on glass coverslips or Transwell inserts were

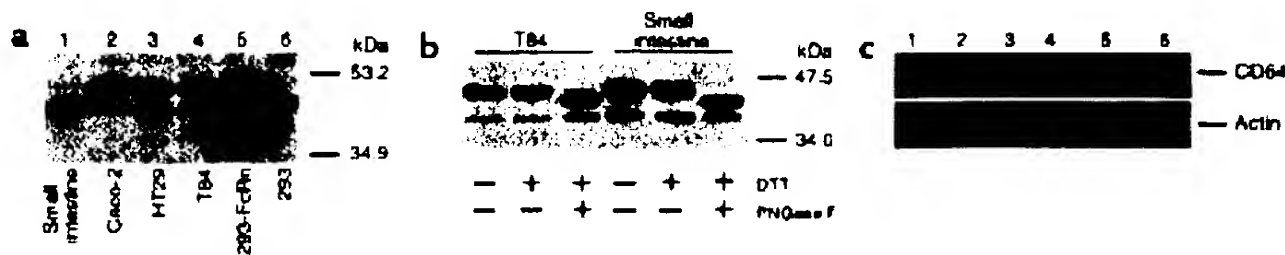


Figure 1

FcRn expression in normal adult human small intestine and human intestinal epithelial cell lines. Western blots of total cellular protein (13 μ g protein per lane, a; 10 μ g protein per lane, b) isolated from the indicated source using affinity-purified rabbit antisera raised against amino acids 112–125 (a) or amino acids 174–188 (b). (c) RT-PCR detection of FcRn transcripts. Total RNA (2 μ g) from T84 (lanes 3 and 4), MOLT-4 (lanes 5 and 6; negative control), and U937 (lanes 1 and 2; positive control) cell lines was incubated with an oligo-dT primer with (odd-numbered lanes) or without (even-numbered lanes) avian myeloblastosis virus-RT (AMV-RT), and a nested PCR was performed with primers specific for FcRn cDNA (top) or for β -actin (bottom).

stained with rhodamine-labeled IgG or were directly fixed in 4% paraformaldehyde, washed in PBS, and stained with the aforementioned anti-FcRn antibodies and in combination with antibodies against tight junction protein ZO-1 (Zymed Laboratories Inc.). Intact monolayers were mounted on glass slides for epifluorescence and/or confocal microscopy. All staining reactions were accompanied by a negative control that consisted of an affinity-purified, isotype-matched nonspecific antiserum. All sections prepared for fluorescent microscopy were mounted in ProLong antifade reagent (Molecular Probes Inc., Eugene, Oregon, USA) and viewed using either a Bio-Rad MRC-1024 confocal microscope or a Zeiss Axiophot microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Height, California, USA). Electronic images were captured and edited in Adobe Photoshop (Adobe Systems Inc., Mountain View, California, USA).

For binding studies, T84 cells grown on glass coverslips were incubated at 4°C with 60 nM IgG-rhodamine in the absence or presence of a 1,000-fold molar excess of underivatized IgG in pH 6.0 or pH 8.0 buffer. Coverslips were processed for epifluorescence microscopy as described previously (23).

Trans epithelial IgG flux studies. Purified human IgG (IgG) and chicken IgG (IgY) (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) were biotinylated with sulfo-NHS-biotin (Pierce Chemical Co., Rockford, Illinois, USA). Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Co.) and confirmed by SDS-PAGE and ligand blot. T84 monolayers exhibiting high electrical resistances (800–1,200 Ω /cm²) were equilibrated in HBSS+, and 60 nM biotin-labeled IgG or IgY (6–600 nM were initially tested) was added to the apical or basolateral reservoirs. As a nonspecific blocker, 0.5% Teleostean gelatin (Sigma Chemical Co.) was included with ligand. In some studies, unlabeled IgG or IgY (30 μ M) or 0.1 mg/mL fragment B of Staphylococcal protein A (Pierce Chemical Co.) was used as a competitive inhibitor. When indicated, T84 cell monolayers were preincubated for 20 minutes with 0.1 μ M bafilomycin A1 (Sigma Chemical Co.) to inhibit

the vacuolar proton pump (29). Monolayers were incubated for 1 hour with ligand at either 37°C or 4°C, after which an aliquot of the contralateral well buffer was collected and 3–4 similarly treated Transwells were combined. Transported proteins were precipitated by acetone or trichloroacetic acid in the presence of 10 μ g yeast tRNA (Boehringer Mannheim GmbH, Mannheim, Germany) and were analyzed by SDS-PAGE and ligand blot after reduction with DTT as described previously (30). In some studies, transported proteins were analyzed by ELISA (see later here). In 1 experiment, NIH Image software (version 1.54; National Institutes of Health, Bethesda, Maryland, USA) was used to determine relative band intensities of a scanned avidin blot in which apically and basolaterally directed IgG transport was analyzed.

ELISA. For analysis by quantitative ELISA, samples were first microconcentrated by centrifugation (Amicon Inc., Beverly, Massachusetts, USA). MaxiSorp 96-well microtiter plates (NUNC A/S, Roskilde, Denmark) were coated overnight at 4°C with 10 μ g/mL of either goat anti-human IgG or rabbit anti-chicken IgY (Jackson ImmunoResearch). Plates were blocked with 1% BSA in PBS/0.05% Tween-20, and samples were added to washed plates and incubated for 1 hour at room temperature or overnight at 4°C. Standards were prepared in duplicate from stock preparations of biotinylated ligands. To detect biotinylated ligands, 0.1 μ g/mL streptavidin-horseradish peroxidase in blocking buffer was added, plates were washed again, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co.) was added as substrate.

Results

Expression of FcRn in adult human intestine and intestinal cell lines. Three intestinal epithelial cell lines and enterocytes purified from normal human adult small intestine were assessed for FcRn protein by Western blot using 2 independently produced antipeptide antisera raised in rabbits (Figure 1, a and b). An affinity-purified rabbit antiserum raised against amino acids 112–125 of human FcRn α 2 domain detected an immunoreactive 45-kDa band in the enterocyte whole-cell lysate and lysates from human T84,

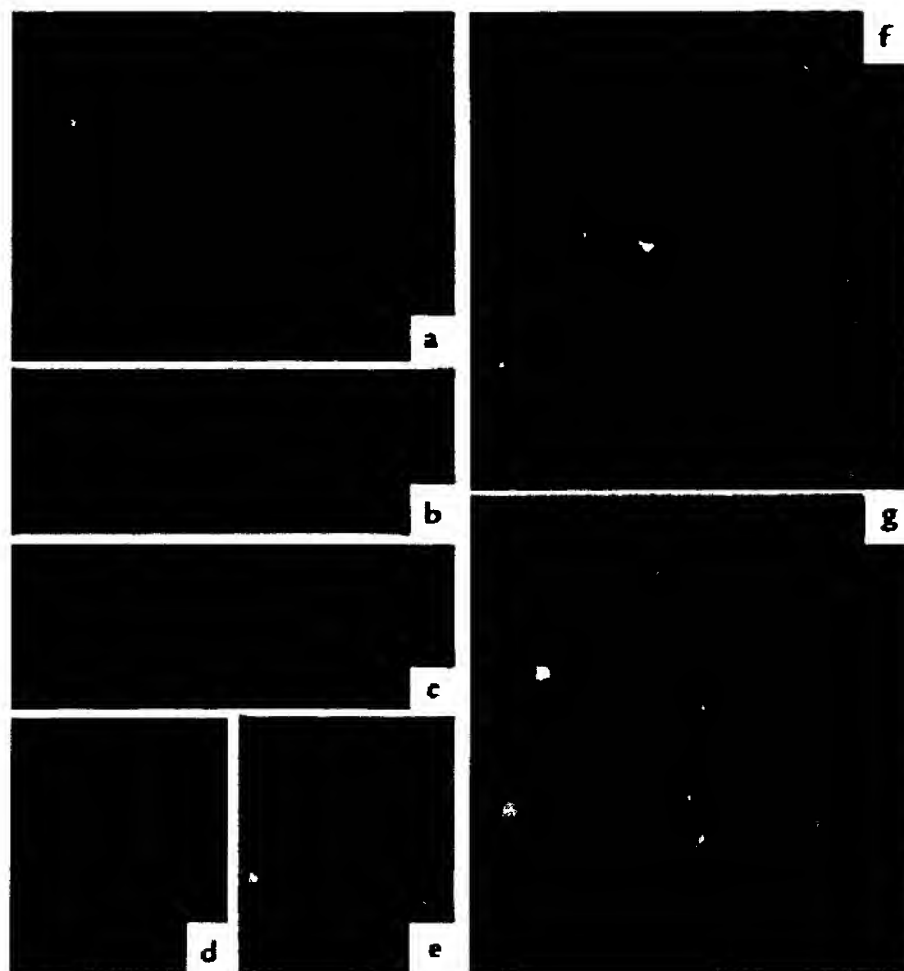


Figure 2

Immunolocalization of FcRn in polarized T84 monolayers (a–c) and normal adult human small intestinal mucosa (d–g). (a) Whole-mount T84 monolayers show a diffuse, punctate staining pattern. The Z0-1 image was captured slightly above the focal plane of FcRn. (b) FcRn staining of whole-mount T84 monolayers viewed as confocal vertical sections. (c) FcRn staining was absent in the presence of an isotype-matched, irrelevant antiserum. (f) Villous enterocytes of normal adult human small intestine show delicate linear staining in the region of the apical cytoplasmic membrane. (g) Crypt enterocytes show an apical and punctate staining pattern visible not only at the level of the apical cytoplasmic membrane, but also in the apical cytoplasm below the level of the apical membrane. (d and e) FcRn staining was absent in the presence of an irrelevant antiserum or with secondary antibody alone (not shown).

HT29, and Caco-2 cell lines (Figure 1a). The human embryonic kidney 293 cell line, transfected (293-FcRn; lane 5) or not transfected (293; lane 6) with full-length human FcRn cDNA cloned from the human placenta (20), served as controls. The 45-kDa band detected is consistent with the known molecular weight of human FcRn heavy chain and migrated with human FcRn α chain expressed in the 293 transfected cells (lane 5). A second band of approximately 48 kDa was recognized by the antibody in protein lysates of cultured human epithelial cell lines only (lanes 2–4). The molecular identity of this band remains unknown but likely represents a cross-reactive protein rather than an altered form of FcRn, as this band was not detected with a second, affinity-purified rabbit antiserum raised against a different epitope of human FcRn heavy chain (amino acids 174–188) (Figure 1b). Using this antiserum, a 45-kDa band corresponding to the FcRn α chain was detected in both T84 cell and

enterocyte whole-cell lysates. The identity of the 45-kDa protein as FcRn was confirmed by the shift in molecular mass after deglycosylation with PNGase F, consistent with the predicted single glycosylation site within the human FcRn α 2 domain. A lower band of approximately 40 kDa was also identified in both T84 cell and human enterocyte lysates with this antiserum. This protein likely represents a cross-reactive protein, as it was neither detected with the antisera directed against amino acids 112–125 (Figure 1a) nor deglycosylated with PNGase F (Figure 1b). These results were verified using enterocytes purified from 3 different adult human small intestinal biopsies (data not shown). Taken together, these data indicate that, like enterocytes purified from the adult human small intestine, the T84, HT29, and Caco-2 cell lines express the 45-kDa glycosylated α chain of FcRn.

To confirm these results, a Northern blot analysis was performed with total RNA obtained from normal adult

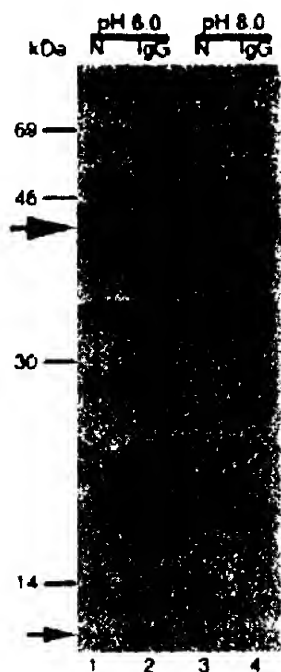


Figure 3

Functional expression of FcRn in polarized T84 cells. T84 cells grown on collagen-coated Transwells were cell surface-biotinylated and solubilized at either pH 6.0 or pH 8.0 lysates were incubated with Sepharose beads (N) or beads coupled to IgG (IgG). Immunoprecipitated proteins were analyzed by SDS-PAGE and avidin blot. A 12-kDa band consistent with the mobility of β_2 M (small arrow) and a 45-kDa band consistent with the mobility of human FcRn α chain (large arrow) were immunoprecipitated by IgG-coupled beads at pH 6.0 but not at pH 8.0.

human small intestinal enterocytes and the human intestinal Caco-2, HT29, and T84 epithelial lines (data not shown). A 32 P-labeled cDNA probe encoding the 3' untranslated region of human placental FcRn cDNA hybridized with a single, abundantly expressed 1.5-kb band in all samples examined. These data were verified by RT-PCR using oligonucleotide primers

specific for the $\alpha 2$ domain of the human FcRn heavy chain, which amplified a 242-bp specific product in the 3 intestinal cell lines and human small intestinal enterocytes (data not shown).

FcRn expression in polarized T84 cells. We selected the T84 cell line for further study based on the results reported here and the ability of T84 cells to form well-differentiated polarized monolayers with high electrical resistances. We determined whether T84 cells express Fc γ RI (CD64), the only other Fc γ receptor that displays high affinity for monomeric IgG (31). As shown in Figure 1c, nested RT-PCR yielded an 800-bp fragment specific for CD64 in the human promonocyte cell line U937 (lane 1, top) but not in polarized T84 cells (lane 3, top) or in the human T-lymphoblast cell line MOLT-4 (predicted to be negative for Fc γ RI; lane 5, top). RT-PCR amplification of human β -actin provided an internal control for each reaction (Figure 1c; lanes 1, 3, and 5, bottom). Thus, the results of IgG flux studies in T84 cells (described later here) were not confounded by cotransport via IgG binding to Fc γ RI.

Expression of human FcRn in polarized T84 cells and normal human small intestine was confirmed by immunohistochemistry using 2 independently raised antibodies (see Methods). T84 cells stained with anti-FcRn antibodies showed a punctate staining pattern (Figure 2a), with the majority of signal close to the apical membrane at or just below the level of the tight junctional ZO-1 expression (Figure 2b). Frozen sections of adult small intestinal mucosa showed distinct villous and crypt staining patterns. Villous enterocytes showed a delicate linear staining pattern at the apical membrane (Figure 2f), which, on tangential sections, appeared to consist of uniform small dots (data not shown). The staining in crypt enterocytes was also apical and punctate, but there was more variability in the size and dis-

tribution of the signal, and positive signal was also identified in the apical cytoplasm below the level of the apical membrane (Figure 2g). In this respect, the T84 staining pattern resembled to a greater extent the pattern seen in crypt enterocytes. Positive immunofluorescence for FcRn was not present in identical T84 monolayers (Figure 2c) or frozen sections (Figure 2, d and e), treated with nonimmune serum and an irrelevant affinity-purified antiserum, respectively. These data corroborate and extend previous evidence that FcRn is expressed in adult human intestine (19), and show that T84 cells express FcRn in a pattern similar to that found in crypt enterocytes of normal human small intestine.

Functional activity on the cell surface. To test the function of FcRn in IgG transport, we first examined the specificity and pH dependency of IgG binding to FcRn solubilized from total T84 cell lysates in vitro. Cell-surface membrane proteins were labeled with biotin, solubilized, and precipitated by Sepharose beads covalently coupled to human IgG using methods modified from previous studies (24). Figure 3 shows that a 12-kDa band consistent with the mobility of β_2 M (small arrow) and a 45-kDa protein consistent with the mobility of human FcRn α chain (large arrow) were precipitated (Figure 3, lane 2). Ligand precipitation by IgG-coupled beads was specific and displayed a pH-dependency characteristic for Fc binding to FcRn: present at pH 6.0 and absent at pH 8.0 (lane 2 vs. lane 4) (13, 20, 32). Nearly identical results were obtained by ligand precipitation of solubilized cell-surface proteins from HT29 cells labeled with 125 I and from Caco-2 cells metabolically labeled with [35 S]methionine (data not shown). The functional integrity of FcRn in T84 cells was confirmed using cells grown on glass coverslips. As assessed by epifluorescence microscopy (Figure 4), binding of rhodamine-labeled IgG to T84 cell surfaces at 4°C was pH-dependent (apparent at pH 6.0 but not at pH 8.0) and strongly reduced in the presence of excess competing unlabeled IgG. When incubated at 37°C, rhodamine-labeled IgG was redistributed into punctate intracellular vesicles consistent with uptake into endosomal vesicles (data not shown).

Receptor-mediated transcytosis of IgG in T84 cells. FcRn-dependent IgG transport was measured by transepithelial flux of biotin-labeled human IgG. Figure 5a shows that after 1 hour at 37°C, intact human IgG (60 nM) applied to apical or basolateral cell surfaces was transported in both luminal and abluminal directions across T84 cell monolayers, as assessed by SDS-PAGE and ligand blot. Transport of heavy (55-kDa) and light (25-kDa) chains of IgG was detected in monolayers incubated at

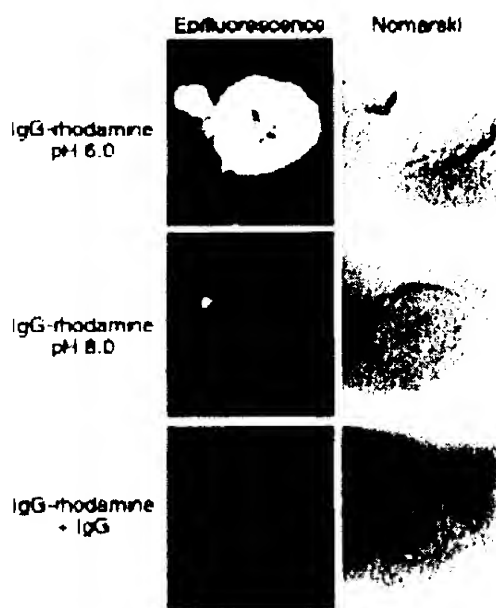


Figure 4

Binding of rhodamine-labeled IgG to T84 cell surfaces. T84 cells grown on glass coverslips were incubated at 4°C with rhodamine-labeled IgG at pH 6.0 or pH 8.0 in the presence or absence of excess competing nonlabeled IgG. Ligand binding was assessed by epifluorescence and Nomarski bright-field microscopy.

37°C (lanes 1 and 3) but not in monolayers incubated at 4°C (lane 2). These data are representative of 7 and 4 independent experiments examining basolaterally directed and apically directed IgG transport, respectively. Thus, transepithelial flux of biotin-labeled IgG did not occur by passive diffusion through intercellular tight junctions or monolayer leaks. These data indicate that IgG can enter both apically and basolaterally directed transcytotic pathways in T84 cells. In a separate experiment like that shown in Figure 5a, the apically directed transport pathway was determined to be approximately 3.6-fold more efficient in IgG transport than the basolaterally directed pathway. In this experiment, band intensities were compared by densitometry and measured against control IgG heavy and light chains (0.5 ng IgG-biotin standard).

Two experimental approaches were used to show that transcytosis of IgG was receptor-mediated and specific for FcRn. First, we used chicken IgY, which is structurally similar to human IgG but does not bind FcRn (33, 34). Apically and basolaterally directed transepithelial transport of human IgG and chicken IgY, both applied at 60 nM, was examined in parallel by avidin blot (Figure 5b) and by quantitative ELISA (Figure 5c). Figure 5b shows that after 1 hour at 37°C, human IgG (60 nM) applied to the basolateral cell surface was transported across monolayers incubated at 37°C (lane 3) but not across monolayers incubated at 4°C (lane 4). In contrast, chicken IgY did not cross T84 cell monolayers in either direction at 37°C (Figure 5b, lanes 2 and 5). A nonspecific avidin-reactive protein band migrating just below the IgY heavy chain was detected in all lanes after TCA pre-

cipitation. This band is a contaminant of the TCA precipitation technique, as it was also present in precipitates containing only the carrier protein, yeast tRNA. These data are representative of 3 independent studies.

We also examined the specificity of basolaterally directed transport in separate studies by quantitative ELISA (Figure 5c). Transcytosis of IgG was detected in monolayers incubated at 37°C but not at 4°C (8.14 ± 2.89 vs. 2.25 ± 0.90 pM/h per cm^2 ; mean \pm SEM), and transport of IgY at 37°C was not significantly different from 4°C controls (1.77 ± 0.93 vs. 0.68 ± 0.18 pM/h per cm^2 ; mean \pm SEM) ($n = 4$; $P = 0.05$ by ANOVA). In addition, we find that horseradish peroxidase, an accepted marker for fluid-phase uptake (non-receptor-mediated endocytosis), is not transported at detectable levels (0.024 pM) across intact T84 monolayers in either direction. Thus, in contrast to the transcytotic routing of FcRn-IgG complexes through the cell, fluid-phase cargo (horseradish peroxidase and IgY) is likely degraded via the lysosomal degradative pathway or is inefficiently transported.

To confirm and extend these results, we examined IgG-biotin transport in the presence and absence of excess native human IgG or chicken IgY as competitive inhibitors. Competition experiments were performed with a 500-fold molar excess of native IgG or IgY (30 μM) (Figure 6, a and b). Data representative of 4 independent experiments are shown. Basolaterally directed transcytosis of IgG-biotin was blocked completely by excess unlabeled IgG (Figure 6a, lane 5), whereas competition with IgY had no effect (lane 4). Incubations at 37°C in the absence of competitive inhibitor (lane 2) and at 4°C (lane 3) provided positive and negative controls. Similarly, excess native IgG (Figure 6b, lane 4), but not IgY (lane 3), blocked apically directed transcytosis of IgG-biotin.

In another version of this approach, IgG transport was inhibited by competition with fragment B of Staphylococcal protein A. Amino acid residues 1253, H310, and H433 — located at the interface between CH2 and CH3 domains of IgG — have been identified by mutagenesis as important for FcRn binding (7). Fragment B of Staphylococcal protein A makes contact with overlapping amino acids in this region (252–254, 308–312, and 433–436; EU numbering) (35) and effectively inhibits IgG binding to FcRn without cross-linking the ligand (9). When used as a competitive inhibitor, fragment B also completely blocked basolateral-to-apical transport of IgG (Figure 6b, lane 5). Competition with excess IgG (lane 4) and IgY (lane 3) provided positive and negative internal controls, respectively. Taken together, these data show that IgG transepithelial transport across T84 cell monolayers is specific and strictly dependent on binding to FcRn.

pH dependency of IgG transport. FcRn is the only Fc receptor that displays pH dependency for binding of IgG. Thus, to confirm the specificity for FcRn in IgG transport and to test the idea that FcRn may first bind its ligand and within the acidic endosome, we examined the pH dependency of IgG transcytosis (Figure 6c). The following studies were performed under conditions in which

the apical and basolateral reservoirs were buffered to pH 8.0 to inhibit IgG binding to FcRn at cell surfaces. To demonstrate pH dependency, T84 cells were incubated in the presence or absence of bafilomycin A1 (0.1 μ M). Bafilomycin A1 is a specific inhibitor of the vacuolar H⁺ ATPase in T84 cells. It collapses pH gradients in intracellular vesicles but does not interfere greatly with membrane trafficking, as evidenced by studies on transferrin receptor recycling in CHO cells (36), receptor-mediated uptake, signal transduction, and transcytosis of cholera toxin and botulinum toxin in T84 cells (29, 37). Figure 6c shows that bafilomycin A1 completely inhibited apical-to-basolateral transport of IgG (lane 2). Incubations at 37° and 4°C in the absence of bafilomycin A1 provided positive and negative internal controls (lanes 3 and 4, respectively). Identical results were obtained in studies on apically directed IgG transport (data not shown). These data are representative of 4 independent studies (2 studies in each direction), and show that FcRn-mediated transepithelial transport of IgG in T84 cells requires the formation of acidic intracellular compartments. However, we cannot exclude the possibility of an indirect effect of bafilomycin A1 on vesicle formation and trafficking. Given the well-known dependence on acidic pH for IgG binding to FcRn, these data provide evidence that IgG may enter the transcytotic pathway by binding to FcRn within acidic intracellular compartments rather than at the cell surface, or that both ligand binding within acidic vesicles and bafilomycin A1-sensitive membrane traffic (38) are required.

Discussion

These studies show that polarized human intestinal T84 cells and normal human adult small intestinal epithelial cells express FcRn. At steady state, FcRn in

T84 cells is localized within apical intracellular vesicles, similar in distribution to that found in crypt enterocytes of native human small intestine. As predicted from the defined function of this molecule in intestinal epithelial cells of suckling mice and rats, we find that FcRn functions in T84 cells to carry IgG across the epithelial barrier by receptor-mediated transcytosis. IgG transport is bidirectional, specific to FcRn, and dependent on endosome acidification. These data define a novel bidirectional mechanism for IgG transport across polarized epithelial barriers.

In the absence of disease, epithelial monolayers lining mucosal surfaces are impervious to solute transport by passive diffusion. This is a fundamental requirement for all transporting epithelia, such as those found in the intestine, lung, and kidney, which function to absorb or secrete solutes and water, often against steep electrochemical and concentration gradients. Thus, very few macromolecules traverse the epithelial barrier intact by passive diffusion (39–42). Some macromolecules, however, may be efficiently transported across epithelial cells by endocytosis into specialized transport vesicles that move across the cell in a process termed transcytosis. This transcellular pathway of macromolecular transport is most often receptor-mediated, as typified by the mechanism for the mucosal secretion of polymeric IgA by the polymeric immunoglobulin receptor (pIgA-R) (43–45), and as described in this report for FcRn-dependent transport of IgG. Macromolecular transcellular transport can occur by fluid phase or non-receptor-mediated endocytosis, but this pathway is inefficient, and most of the endocytosed cargo is delivered to the lysosome for degradation (46).

The mechanism of FcRn-mediated IgG transport apparent in T84 cells, however, differs from that defined

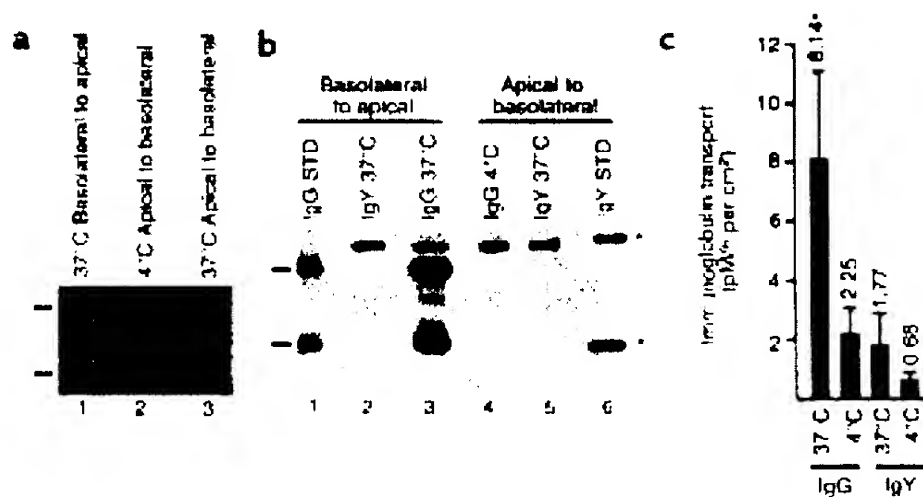


Figure 5

FcRn-dependent bidirectional transcytosis of IgG across T84 cell monolayers. (a) Transcytosis of IgG-biotin occurs at 37°C (lanes 1 and 3) but not at 4°C (lane 2). (b) Specificity of IgG transport for FcRn. T84 cells transport human IgG-biotin at 37°C (lane 3) but not at 4°C (lane 4). IgG heavy and light chains are indicated by the bars to the left of the IgG standard. In contrast, chicken IgY-biotin does not cross T84 cell monolayers at 37°C in either direction (lanes 2 and 5). IgY heavy and light chains are indicated by asterisks to the right of the IgY standard. (c) Quantitative ELISA of IgG and IgY transport at 37°C and 4°C.

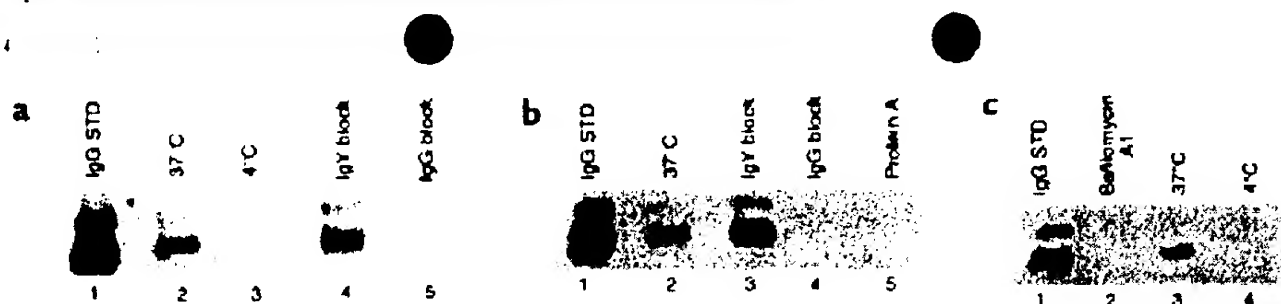


Figure 6

Specificity of IgG transport and functional dependence on the vacuolar H^+ ATPase. Where transport occurred, the 25-kDa biotinylated light chain of IgG is shown. (a) Apical-to-basolateral transcytosis of IgG-biotin (60 nM) in the absence or presence of a 500-fold molar excess of nonlabeled chicken IgY (30 μ M) or human IgG (30 μ M) (lanes 4 and 5, respectively). Lanes 2 and 3 show incubation in the absence of competitive inhibitor at 37°C and 4°C as positive and negative controls, respectively. (b) Basolateral-to-apical transcytosis of IgG-biotin (60 nM) in the absence or presence of a 500-fold molar excess of nonlabeled chicken IgY (30 μ M) (lane 3) or human IgG (30 μ M) (lane 4) or excess fragment B of Staphylococcal protein A (0.1 mg/mL; lane 5). Lane 2 shows incubation at 37°C as a positive control. (c) T84 cells were incubated in the presence (lane 2) or absence (lanes 3 and 4) of bafilomycin A1 (0.1 μ M), and basolaterally directed transport of IgG-biotin (60 nM) was measured after 1 hour of incubation in symmetrical HBSS+ buffered to pH 8.0.

for the pIgA-R. First, transcytosis by FcRn is bidirectional, as shown in this report. Second, unlike the pIgA-R, FcRn does not undergo proteolysis after delivering its ligand to the apical cell surface. Our data suggest instead that FcRn may reenter the cell for return to the opposite membrane. Thus, unlike the pIgA-R, which is committed to only 1 round of dimeric IgA transport, the same FcRn molecule may transport IgG for multiple rounds in both luminal and abluminal directions. Third, unlike the pIgA-R, FcRn may initially bind its ligand within acidic endosomes rather than at the cell surface. This concept is not new. In contrast to the rat and mouse neonatal intestine, where maternal IgG binds FcRn at the apical cell surface (47), FcRn expressed in the rat yolk sac appears to bind IgG only within apical compartments after fluid-phase uptake (48). Also, Benlounes et al. (49) have observed transepithelial IgG transport across isolated neonatal rat intestine in which both the serosal and mucosal solutions were clamped at neutral pH and thus inhibitory for IgG binding to FcRn at the cell surface.

Several lines of evidence support the idea that FcRn, like the pIgA-R, may function in vivo to transport immunoglobulins across epithelial barriers. First, IgG is present in secretions of the human oral mucosa, small and large intestine, lung, and genitourinary tract (50-53). The mechanism by which IgG is transported onto these mucosal surfaces is unknown. Some evidence suggests that luminal IgG represents passive transudation from the serum. This view is challenged by studies that show that numbers of local IgG-secreting cells in mucosal tissues and levels of antigen-specific IgG in mucosal secretions can be dramatically increased after both mucosal and systemic immunization (54-60). In addition, Berneman et al. (57) have found that the patterns of IgG subclasses in human serum differ from those found in autologous secretions at mucosal surfaces, and demonstrate that the concentration of IgG subclasses differ from those found in the serum. These data provide evidence that IgG in luminal secretions may be derived from local origin and transported selectively across mucosal barriers. Whether

FcRn mediates IgG transport in the adult human intestine, however, has not been tested directly.

On the other hand, the results of our in vitro studies on IgG transport across T84 cell monolayers predict that FcRn may function in vivo to establish and maintain a steady-state distribution of IgG across the epithelial barrier of the human intestine, and possibly across other mucosal surfaces that bear FcRn. Such a putative function would have important effects on IgG-mediated mucosal immunity and host defense by providing a mechanism for antigen transport to subepithelial lymphocytes in the absence of a disrupted epithelial barrier. We can envision that FcRn may deliver IgG across the epithelial barrier for recognition of cognate antigens in the lumen, and then may function in the opposite direction for delivery of immune complexes to lymphocytes in the lamina propria. In this regard, it is already well known that FcRn can transport antigen-antibody immune complexes across the intestine of suckling rodents (33). Thus, FcRn may effectively mediate a novel mechanism for immunosurveillance and host defense at mucosal sites.

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